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Spectral markers and morphological features of diabetic foot ulcer tissues

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Metabolic disorders are an integral part of the development of diabetes mellitus. One of the most severe complications of diabetes is a diabetic foot, which is characterized by the formation of necrotic sores. The aim of the study was to experimentally test the hypothesis of the formation and accumulation of β-structured protein aggregates in diabetic foot tissues as markers of diabetes associated disorders. The formation of β-structured protein aggregates promotes their non-enzymatic glycosylation, which in turn promotes aggregation. There is a positive connection between the individual links of the pathological process. Breaking this link is a prerequisite for effective treatment. The results of the optical image analysis indicate the presence of β-structured protein aggregates, confirmed by IR spectroscopy. Thus, the presence of the total β-conformation reaches 47,6%. The assumption of an increasing contribution from the β-antiparallel conformation was made. Clear IR markers of necrotic tissues in the absorption region of stretching vibrations of C=O and C–O molecular groups as well as a 2.5-fold increase in the intensity of the CH stretching bands compared to OH were observed. Conformational fitting and correlation analysis between different protein phases showed that the necrosis spectroscopic feature strongly correlates with water content, and α-helix plus triple helix negatively correlate with water content, β-conformation, and necrosis features. The assumption of an increasing contribution from β-antiparallel conformation connected with amyloid-like fibril formation was done.

Keywords: diabetes, diabetic foot, metabolic disorders, protein aggregation, β-conformation, water content, necrosis marker, phase correlation, FTIR spectroscopy.

1. Introduction

Diabetes mellitus is a chronic degenerative disease that occurs when the pancreas does not produce enough insulin, or the body cannot use it effectively, and is accompanied by numerous complications. The development of oxidative stress and the accumulation of carbonyl compounds causes non-enzymatic glycosylation of proteins (glycation). Violation of the structure and functioning of vessels contributes to the local accumulation of abnormal metabolites, in particular, structurally damaged proteins. It is known that non-functional glycosylation contributes to protein aggregation and vice versa, destabilized and aggregated proteins become much more vulnerable to non-enzymatic glycosylation. Such aggregated structures are insoluble, resistant to proteolysis, immunogenic, and capable of sorption and conformational rearrangement of soluble proteins in their own way. An extreme case of these processes is the formation of amyloid structures.

Uncontrolled hyperglycemia is considered the main cause of diabetic complications, as it leads to the formation

of advanced glycosylation end products (AGEs). AGEs are formed as a result of the classic Maillard reaction, i.e., a reaction between proteins and sugars, which can occur not only during food preparation but also in a living organism. Under normal conditions, the reaction rate is so slow that its products have time to deactivate. However, in persistent hyperglycemic states, due to increased glucose availability, the reaction is significantly accelerated, and the products accumulate. As a result, the level of damaged proteins increases (for example, the concentration of glycosylated hemoglobin, which is a marker of diabetes) [1–3].

These processes are schematically presented in Fig. 1. Sugars react non-enzymatically with the amino group of proteins, lipids, and nucleic acids through a series of reactions, forming a Schiff base, followed by Amadori rearrangement and further oxidative modifications (glycoxidation) with the formation of AGEs. (Fig. 1).

The carbonyl group of sugars can react, for example, with free terminal amino groups of the amino acid lysine, which is very abundant in collagen. One of the common late products of the Maillard reaction is carboxymethyllysine, a derivative of lysine. Carboxymethyllysine in proteins is a biomarker of general oxidative stress in the body. It accumulates in tissues, for example, in skin collagen, and is increased in diabetes.

The formation of structurally damaged proteins is an integral circumstance of processes associated with tissue degradation processes. Due to the imbalance of the structure, such derivatives are vulnerable to non-enzymatic glycosylation and aggregation. They become secondary toxins that disrupt the normal functioning of surrounding tissues. These processes are most pronounced in the local accumulation of tissue breakdown products, which is a typical circumstance of the so-called diabetic foot, namely progressive ulcer of the lower extremities of patients with diabetes [5, 6].

More than 100 physiological factors are involved in the healing process, and some of them are impaired in people

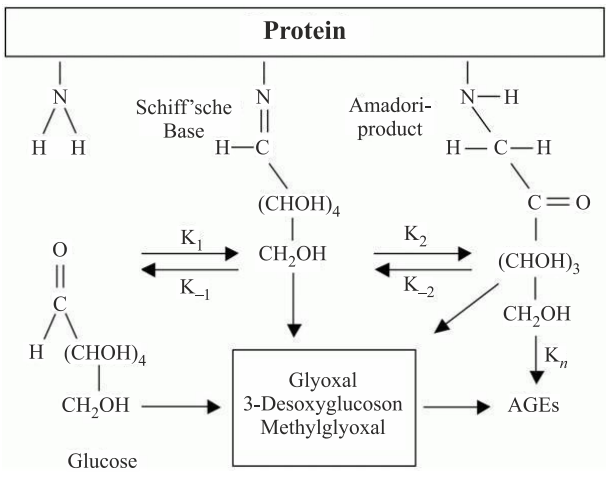

Fig. 1. Schematic representation of the processes of non-enzymatic protein glycosylation in diabetes mellitus [4].

with diabetes, leading to poor wound healing. These include reduced production of growth factors, changes in angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, amount of granulation tissue, migration and proliferation of keratinocytes and fibroblasts, number of epidermal nerves, bone healing, and an imbalance between the accumulation of extracellular matrix (ECM) components and their remodeling by matrix metalloproteinases (MMPs) [7–9]. Therefore, it is important to analyze the healing process at the molecular level using accurate and rapid methods, and to investigate and propose effective treatments that improve healing or enhance skin regeneration. In this sense, infrared spectroscopy is a valuable tool for studying biomolecules and tissues, as it provides information on the molecular structure of organic and inorganic materials. Vibrational spectra are sensitive to conformational analysis of the secondary structure of proteins, examining bands corresponding to α-helices, β-sheets, β-turns, and disordered structure [10, 11]. The aim of this work was to conduct a comprehensive study of diabetic foot tissues using modern biophysical methods. As a result of the study, the presence of β-structured protein aggregates in the studied tissues was proven, which is a significant component of the complications of diabetes mellitus.

2. Materials and methods

Samples of operative material of pathological tissues of the diabetic foot with fixation in 10% formaldehyde solution were studied. Samples were collected in accordance with the provisions of bioethics with the consent of the patients. The structure of the studied histological samples was evaluated using Congo red staining according to Puchtler–Meier [12, 13]. The infrared spectra of the experimental samples were recorded using TENSOR 27 (Bruker) instrument with attenuated total reflection (ATR) attachment. The wavenumber accuracy was about 0.01 cm^{-1}, and the absorbance accuracy was 0.1%. All spectra were collected using 32 scans in the range from 3800 to 400 cm^{-1}. To record the spectra, a small area of tissues was washed from formaldehyde, then applied to the diamond crystal of the ATR and left to dry at room temperature.

Before recording the spectra, the ATR crystal was cleaned with ethanol and allowed to dry. All registered spectra were processed using the OPUS 8.2 software (Bruker). Amide I band was deconvoluted using the PeavzFit v4 program.

Experimental diabetic foot tissue samples S2 and S3 are pathological but characterized by different levels of necrosis. The samples, labelled black part (bp) and white part (wp), were collected directly at the site of the diabetic ulcer, light (wp) and dark (bp) parts of the ulcer tissues. Other samples were taken at a distance from the immediate ulcer.

The confocal images were obtained on a laser scanning confocal microscope Carl Zeiss (Germany) LSM 510 META. The research was carried out in three modes: in the light microscope mode (in the light of a halogen lamp, which

illuminated the sample), in the fluorescent microscope mode (in the light of an ultraviolet mercury lamp HBO-103 W/2, which excited the fluorescence of dyes), as well as in the confocal microscope mode. A Plan-Apochromat 63x/1.30 Oil DIC objective was used to obtain images of the experimental samples.

3. Results and discussion

3.1. Microscopic studies

When staining diabetic foot tissues with Congo red, the presence of β-structured protein aggregates was detected in their composition. They are stained in various shades of brick-red in a light microscope [Figs. 2(a)–2(c)] and apple-green in a polarizing microscope [Figs. 2(d)–2(f)]. It is worth emphasizing that the similarity of Congo red staining of fibrous and oncological tissues to the case of amyloids is sometimes interpreted as evidence of insufficient selectivity of this dye as specific only to amyloids [14]. However, in the context of determining the structure of amyloids like β-structured protein aggregates elucidating the exceptionally high energetic benefit of their formation [15, 16], such a statement seems unfounded.

The presence of amyloid-like structures in various pathological tissues was shown using a number of dyes

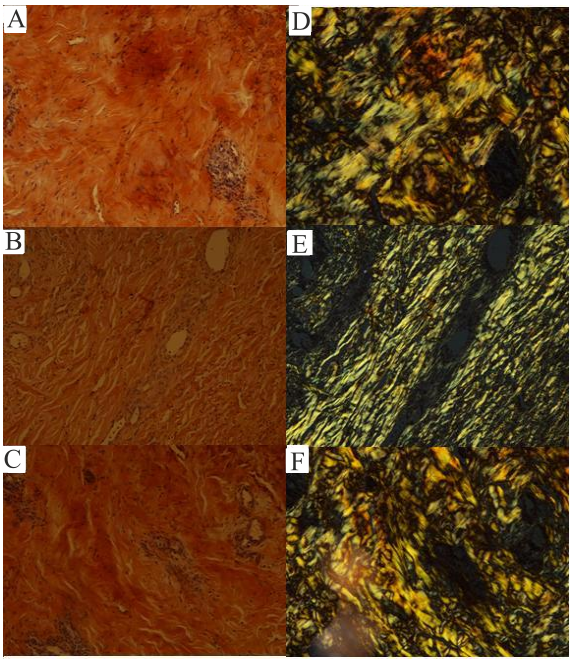

Fig. 2. (Color online) The microscopic images of the diabetic foot tissues. Congo red staining, magnification × 200 times. A, B, and C are transmittance mode. The fibrous structure of the tissue is visible but the fibres are twisted, indicating damage. There are many cells and cell clusters in the field of view, probably leukocytes, their nuclei are dark in color, indicating an active phase of inflammation. D, E, and F are polarizing microscopy. The bright green color of the image indicates the presence of β-aggregates in the tissue.

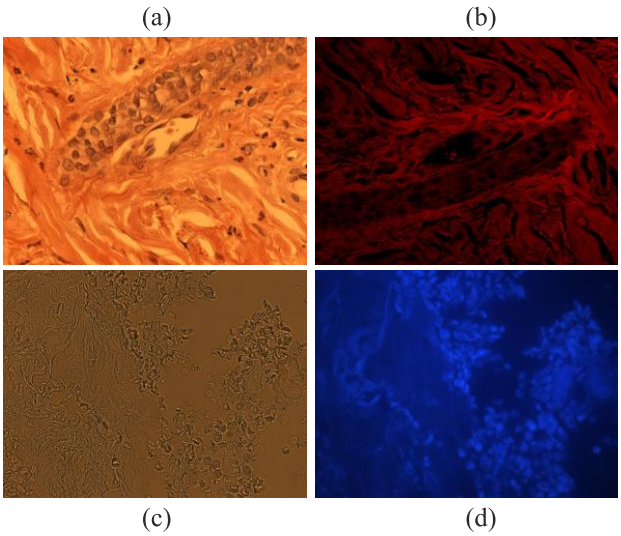

Fig. 3. (Color online) Fluorescent images of diabetic foot tissue. Congo red staining, transmittance mode (a), Congo red staining, excitation on 543 nm (b), thioflavin staining, transmittance mode (c), thioflavin staining, excitation on 405 nm (d).

long before the introduction of Congo red into scientific research. For the above reasons, Congo red should be recognized as a dye specific for β-structured protein aggregates, the latter of which can be both amyloids and other structures formed as a result of impaired protein metabolism [17].

The formation of β-structured protein aggregates promotes their non-enzymatic glycosylation, which in turn promotes aggregation. [18]. There is a positive connection between the individual links of the pathological process. The rupture of this link is a necessary condition for effective treatment. In this case, it is worth paying attention to such an approach to normalizing metabolism as enzymatic cleaning of the wound surface using proteolytic enzymes [19].

Figure 3 shows confocal images of diabetic foot tissue samples. The sample stained with Congo red (a), (b) also shows a heterogeneous fibre structure and cells in the field of view. The thioflavin stained samples (c), (d) have an even more damaged structure, and the cells in the field of view, due to their large size, rounded shape, and single large nucleus, may be macrophages, indicating the presence of dead tissue. In pathological contexts, the presence of macrophages could indicate the presence of chronic inflammation, tissue damage, or infection. Thioflavin is also a fluorescent dye commonly used to stain amyloid fibrils and β-aggregates, where it helps to highlight pathological protein deposits under fluorescence microscopy [11].

3.2. IR spectroscopy studies

The main molecular components of the skin tissue of the foot are proteins (collagen, elastin), therefore the IR absorption spectra of diabetic foot tissues have a characteristic appearance for protein spectra. The main bands of the IR spectra of proteins are the absorption bands of the amide

group, i.e., the peptide bond –CO–NH–. These are amide A (3280 cm^{-1}), amide B (3083 cm^{-1}), amide I (1645 cm^{-1}), amide II (1540 cm^{-1}), amide III (1238 cm^{-1}). The IR bands of amides A and B are sensitive to hydrogen bonds, the presence of hydrogen bonds causes a significant decrease in frequencies and an increase in the half-width of the bands.

The amide I and amide II bands are characteristic and have clearly defined frequencies for the α-helix, β-form and disordered form of the polypeptide chain, which makes it possible to conduct a conformational analysis of the secondary structure of the protein. [20, 21]. The bands of stretching vibrations of the molecular groups CH_3 (2950 and 2880 cm^{-1}), CH_2 (2920 and 2850 cm^{-1}), deformation vibrations CH (1480–1330 cm^{-1}), C=O (1740 cm^{-1}) also are characteristic. The assignment the main IR absorption bands is presented in Table 1.

As for the amide III band in the region of 1240 cm^{-1}, it is characteristic for the analysis of the structure of collagen. The article [22] reports the possibility of detecting collagen in tissues using the bands at 1204, 1240, 1284, and 1338 cm^{-1}. The contribution to the absorption of the amide III band at 1240 cm^{-1}, together with protein components, is given by asymmetric vibrations of the molecular group PO_2^- of phospholipids and nucleic acids, however, the intensity of the shoulder at 1204 cm^{-1} allows conclusions to be drawn about the collagen structure.

Clear markers of necrosis were established in the IR absorption spectra of a diabetic foot ulcer. The first is an increase in the contribution of the shoulder in the region of 3450 cm^{-1}, which refers to the OH stretching vibrations, as well as a sharp increase in the intensity of the CH contribution of the stretching vibrations. The position of the main maximum of the amide A band undergoes a high-frequency shift when moving to tissues with a greater contribution of necrosis (from 3278 to 3290 cm^{-1}). It is also possible to note the redistribution of contributions of CH_2/CH_3 components in the direction of growth of CH_2 with a high degree of tissue necrosis [Fig. 4(a)].

Table 1. Assignment of the main IR absorption bands of diabetic foot tissue (in cm^{-1})

S2-11	S2-6	S3-1	S3-bp	S3-wp	Assignment
3460	3450	3490	3450	3476	O–H str
3283	3278	3275	3289	3290	amid A, Fermi resonance of N–H str with the amide II overtone
3083	3081	3065	3083	3083	amid B, Fermi resonance of N–H str with the amide II overtone
		3008	3003	3006	CH str
2956	2955	2956	2955	2955	$C–H_3$ str
2923	2922	2923	2924	2924	$C–H_2$ str
2872	2872	2873	2870	2871	$C–H_3$ str
2852	2852	2853	2854	2854	$C–H_2$ str
1741	1744	1744	1743	1741	C=O
1635	1642	1645	1639	1639	amid I, α-helix (C=O str (80%), C–N str (10%), N–H def (10%), triple helix
1540	1539	1534	1540	1543	amid II, C–N str (40%), N–H def (60%)
1453	1452	1452	1460	1452	$C–H_2$ def
			1416		C–H def
1384	1386	1383	1378	1381	$C–H_3$ def
1337				1339	$C–H_3$, $C–H_2$ def
1312	1310	1310	1312	1312	$C–H_3$, $C–H_2$ def
1283	–	1283	1279	–	amid III, N–H def
1237	1238	1236	1238	1236	amid III, N–H def
1203	–	1203	–	–	amid III, N–H def
1162	1169	1167	1162	1165	C–O
1118	1115	1115	1115	1118	C–O
1087	1090	1078	1090	1078	ν(C–O–C)
1032	1030	1038	1031	1036	C–O
970	971	968	973	971	C–O, C–C
936	935	935	936	936	C–O, C–C

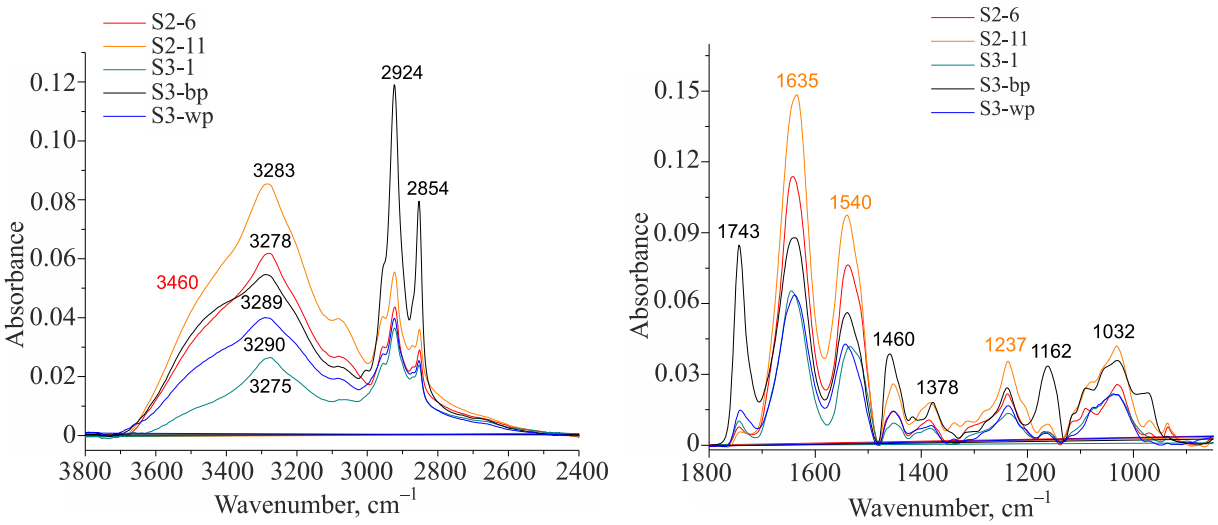

Fig. 4. (Color online) FTIR–ATR spectra of the diabetic foot tissue in the region of (a) 3800–2400 cm^{-1} and (b) 1800–850 cm^{-1}.

Another characteristic marker of necrosis is the C=O band in the region of 1741 cm^{-1}. Both protein and lipid components contribute to the absorption of this area. In the S3-bp sample, the integrated intensity of this band is almost 40% of the intensity of the amide I band [Fig. 4(b)].

The third region characteristic of necrosis is 1160 cm^{-1}, which refers to C–O stretching vibrations. All these spectral markers indicate the processes of tissue destruction accompanied by glycosylation of proteins.

The amide I band, despite its seemingly simple shape, has a very complex nature and represents an overlap of contributions from various secondary structures of protein components of the studied tissues. Conformational analysis of this region allows us to draw conclusions about changes in the secondary structure of proteins. For the decomposition into components of this band, we chose the following model: α-helix and triple helix together named as helix (1650–1655 cm^{-1}), disordered structure (1645–1652 cm^{-1}), parallel β-sheets (1628–1635 cm^{-1}, 1680–1690 cm^{-1}), antiparallel β-sheets (1620–1624 cm^{-1}, 1690–1696 cm^{-1}), turns (1670–1675 cm^{-1}), side groups (1608–1619 cm−1), and deformation vibrations of water (1637 cm^{-1}). Some articles note the presence of a contribution of the collagen triple helix in the region 1638 cm^{-1} [23, 24]. However, these works were carried out on model systems and purposefully excluded the contribution of water. In the case of studying real tissues, the contribution of OH deformation vibrations in this region plays a decisive role, so we focused our attention on this interpretation. The band in the region 1740 cm^{-1} was attributed to the stretching vibrations of C=O, which has a direct correlation with the processes of necrosis in tissues [Figs. 5(a)–5(c)]

Component analysis of the amide I band (Table 2, Figs. 6 and 7) shows a clear correlation between the degree of necrosis in the studied tissues and the contribution of various components to this band. In the sample with the greatest contribution to necrosis (S3-bp), we see a decrease in the contribution of the helix, an increase in the contribution of the β-structure, and an increase in the contribution of OH, which directly correlates with the region of OH stretching vibrations (band 3450 cm^{-1}).

The Pearson correlation coefficient r_{xy} between variables x and y was calculated as the ratio between the covariance of two variables and the product of their standard deviations [25]:

$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}},$$

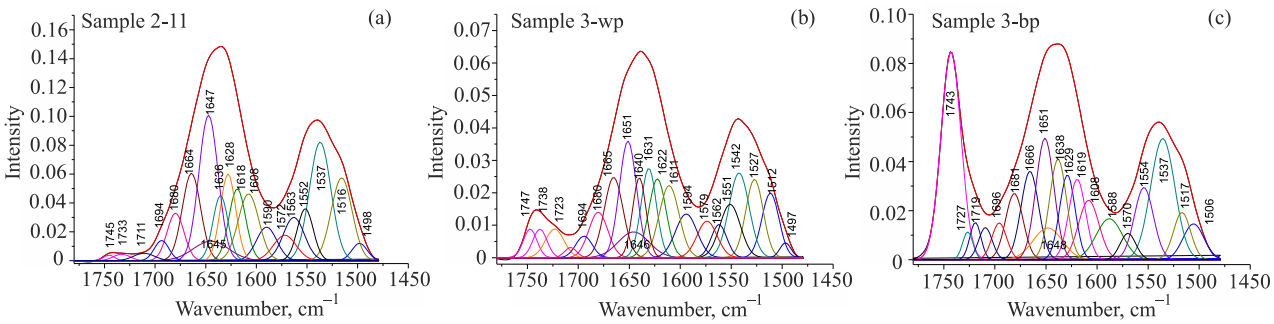

Fig. 5. (Color online) Component analysis of the amide I band.

Table 2. Conformational analysis of the amide I band. The contribution of different components in percentages (%) is indicated (see Fig. 6)

Samples name	Necrosis	Side	Antiparallel β-sheets	Parallel β-sheets	H_2O	Disordered	Helix	Turns
S2-11	2.13	11.96	12.96	18.64	8.51	6.08	24.32	13.88
S2-6	3.76	12.91	12.5	17.53	7.2	6.25	25.68	13.91
S3-1	7.63	11.86	12.59	18.16	7.26	8.72	24.7	12.59
S3-BP	39.91	9.46	14.98	19.56	13.41	7.26	17.19	12.15
s3-WP	12.22	12.62	15.19	19.86	9.58	7.01	17.76	13.32

where n is the sample size, $\bar{x}$ and $\bar{y}$ are mean values for variable x and y, respectively.

The correlation coefficients for 5 samples were calculated for each pair of variables and represented in Table 3. The helix fraction negatively correlates with the β-conformation, water, and necrosis fractions. The necrosis fraction has a strong positive association with water. No correlations were observed between the disordered and beta phases. Expectedly, parallel and antiparallel beta phases are highly correlated.

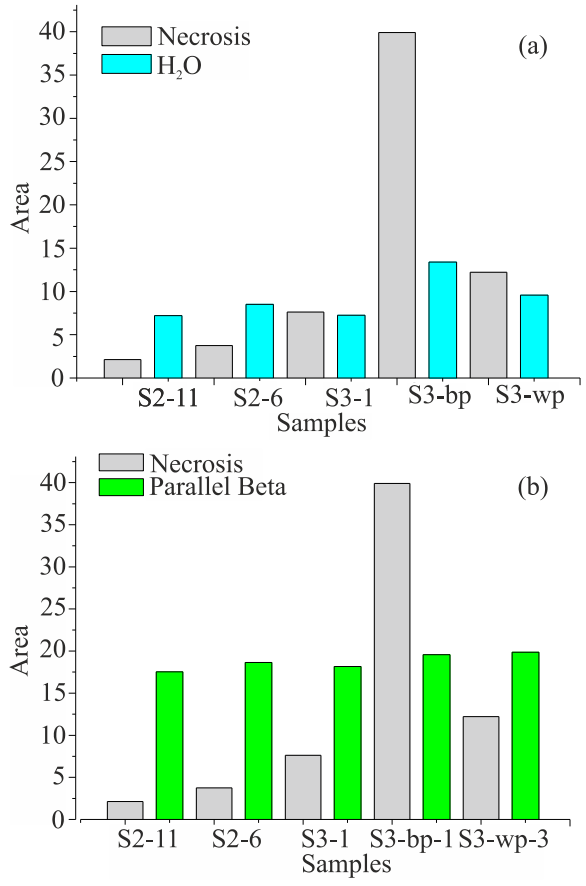

Fig. 6. (Color online) Comparison of the contribution of necrosis at 1742 cm^{-1} and OH deformation vibrations at 1638 cm^{-1} (a) and the contribution of necrosis and parallel β-structures (b). The intensity in the diagram reflects the contribution of the integrated intensity in percentages of the corresponding component relative to the total integrated intensity of the amide I band.

4. Conclusions

1. Data of IR spectroscopy and microscopic images support the idea of the presence of β-structured protein aggregates in diabetic foot tissue. The increase of β-parallel and antiparallel phases in necrotic samples is almost the same and amounts to 2–3% for each. Confocal microscopy images indicate the formation of amyloid-like fibrils that are in accordance with IR spectroscopy data on increasing the β-antiparallel phase.

2. The decrease of helix conformation from 25.68 to 17.19% along with a slight decrease of side groups from 12.62 to 9.46% and turns from 13.91 to 12.15% as well as an increase of total β-structures (turns including) up to 47.67% and increase of water content from 7.2 to 13.41% (necrosis points) have been observed.

3. We observed a 5-time increase in the intensity of C=O stretching vibration in necrotic localization of foot tissue and a 2.5-fold increase in the intensity of the CH stretching modes relative to OH stretching mode, and they can be considered as markers of necrosis.

4. The necrosis spectroscopic feature strongly correlates with water content and anticorrelate with α-helix plus triple helix conformations, turns, and antiparallel β-conformation.

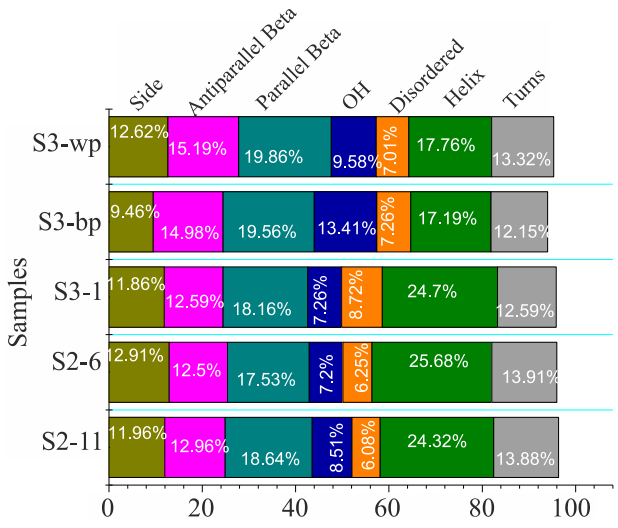

Fig. 7. (Color online) The contribution of different components in percentages (%) for amid I band.

Table 3. The correlation matrix between the secondary structure states, side groups, water, and necrosis

	Parallel	Antiparallel	Beta*	Alpha	Disordered	Turns	Side groups	Water	Necrosis
Parallel β	1.00	0.95	0.98	–0.94	0.05	–0.43	–0.45	0.76	0.61
Antiparallel β	0.95	1.00	0.99	–0.99	0.01	–0.45	–0.46	0.81	0.71
β-phase	0.98	0.99	1.00	–0.98	0.03	–0.44	–0.46	0.80	0.68
Helix	–0.94	–0.99	–0.98	1.00	–0.09	0.55	0.55	–0.86	–0.79
Disordered	0.05	0.01	0.03	–0.09	1.00	–0.75	–0.26	–0.01	0.23
Turns	–0.43	–0.45	–0.44	0.55	–0.75	1.00	0.80	–0.64	–0.81
Side groups	–0.45	–0.46	–0.46	0.55	–0.26	0.80	1.00	–0.86	–0.90
Water	0.76	0.81	0.80	–0.86	–0.01	–0.64	–0.86	1.00	0.95
Necrosis	0.61	0.71	0.68	–0.79	0.23	–0.81	–0.90	0.95	1.00

*β-phase is defined as the sum of parallel and anti-parallel and Helix as sum of α-helix and triple helix.

The α-helix plus triple helix anti-correlate with water content, β-conformation, and necrosis features. Increased water content with increase total β-conformation, decrease turns, side groups, and helix conformations could be considered as marker of diabetic foot ulcer tissue. The appearance of additional β-structures as well as hydroxylation seems to start from side groups, and it is accompanied by rearrangement in helix and β-conformation including all types of turns.

5. The data obtained raises the question of the necessary methodological approaches to prevent the progression of sores and requires further research to develop effective therapeutic strategies.

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Спектральні маркери та морфологічні особливості тканин діабетичної стопи

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Порушення обміну речовин є невід'ємною частиною розвитку цукрового діабету. Одним з найважчих ускладнень цукрового діабету є діабетична стопа, яка характеризується утворенням некротичних виразок. Метою роботи була експериментальна перевірка гіпотези про утворення та накопичення β-структурованих білкових агрегатів у тканинах діабетичної стопи. Утворення β-структурованих білкових агрегатів сприяє їх неферментативному глікозилюванню, яке, у свою чергу, сприяє агрегації. Існує позитивний зворотній зв'язок між окремими ланками патологічного процесу. Розрив цього зв'язку є обов'язковою умовою ефективного лікування. Результати аналізу оптичних зображень вказують на наявність β-структурованих білкових агрегатів, що підтверджено ІЧ-спектроскопією. Таким чином, наявність сумарної β-конформації досягає 45%. Крім того, спостерігались чіткі ІЧ-маркери некротичних тканин в області поглинання валентних коливань молекулярних груп C=O та C–O, а також 2,5-разове збільшення інтенсивності смуг валентності CH порівняно з OH. Конформаційна підгонка та кореляційний аналіз між різними білковими фазами показали, що спектроскопічна характеристика некрозу сильно корелює з вмістом води, а α-спіраль і потрійна спіраль негативно корелюють із вмістом води, β-конформацією та особливостями некрозу. Було зроблено припущення про збільшення внеску β-антипаралельної конформації, яка пов'язана з утворенням амілоїдних фібрил.

Ключові слова: цукровий діабет, діабетична стопа, метаболічні порушення, білкова агрегація, β-конформація, вміст води, маркер некрозу, фазова кореляція, FTIR спектроскопія.